

Chronic methylphenidate treatment during adolescence has long-term effects on monoaminergic function.

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Abstract:	<p>Psychostimulants like methylphenidate or D-amphetamine are often prescribed for attention deficit and hyperactivity disorders in children. Whether such drugs can be administered into a developing brain without consequences in adulthood is still an open question. Here, using in vivo extracellular electrophysiology in anaesthetised preparations, combined with behavioural assays, we have examined the long-term consequences in adulthood of a chronic methylphenidate oral administration (5 mg/kg/day, 15 days) in early-adolescent (Post Natal Day 28) and late-adolescent (PND 42) rats, by evaluating body weight change, sucrose preference (indicator of anhedonia), locomotor sensitivity to D-amphetamine and electrical activities of ventral tegmental area (VTA) dopamine and dorsal raphe nucleus (DRN) serotonin neurons. Chronic methylphenidate treatment during early or late adolescence did not induce weight deficiencies and anhedonia-like behaviours at adulthood. However, it increased bursting activities of DRN serotonin neurons. Furthermore, chronic methylphenidate treatment during early but not during late adolescence enhanced D-amphetamine-induced rearing activity, as well as VTA dopamine cell excitability (firing, burst and population activity), associated with a partial desensitisation of dopamine D2 auto-receptors. We have demonstrated here that early, but not late, adolescent exposure to oral methylphenidate may induce long-lasting effects on monoamine neurotransmission. The possible clinical implication of these data will be discussed.</p>

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Title

Chronic methylphenidate treatment during adolescence has long-term effects on monoaminergic function.

Short Title

Long-term effects of methylphenidate

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Abstract

Background Psychostimulants like methylphenidate or D-amphetamine are often prescribed for attention deficit and hyperactivity disorders in children. Whether such drugs can be administered into a developing brain without consequences in adulthood is still an open question.

Methods Here, using *in vivo* extracellular electrophysiology in anaesthetised preparations, combined with behavioural assays, we have examined the long-term consequences in adulthood of a chronic methylphenidate oral administration (5 mg/kg/day, 15 days) in early-adolescent (Post Natal Day 28) and late-adolescent (PND 42) rats, by evaluating body weight change, sucrose preference (indicator of anhedonia), locomotor sensitivity to D-amphetamine and electrical activities of ventral tegmental area (VTA) dopamine and dorsal raphe nucleus (DRN) serotonin neurons.

Results Chronic methylphenidate treatment during early or late adolescence did not induce weight deficiencies and anhedonia-like behaviours at adulthood. However, it increased bursting activities of DRN serotonin neurons. Furthermore, chronic methylphenidate treatment during early but not during late adolescence enhanced D-amphetamine-induced rearing activity, as well as VTA dopamine cell excitability (firing, burst and population activity), associated with a partial desensitisation of dopamine D₂ auto-receptors.

Conclusions We have demonstrated here that early, but not late, adolescent exposure to oral methylphenidate may induce long-lasting effects on monoamine neurotransmission. The possible clinical implication of these data will be discussed.

Key words-Methylphenidate, Adolescence, dopamine neurons, serotonin neurons, electrophysiology

Introduction

Attention deficit hyperactivity disorder (ADHD) is a common psychological disturbance which affects an average of 5% of school-aged children worldwide (with very variable rate of prevalence ranging from 1 to 20%) (Polanczyk et al., 2015). It is characterised by inattention, impulsivity and hyperactivity. Currently available ADHD treatments include psychostimulants, such as D-amphetamine (D-AMP) and methylphenidate (MPH), which when administered at the adequate dose, have a powerful and immediate therapeutic effect. However, as indirect dopamine agonists, they also have the potential to affect the motor system and disrupt dopamine-dependent behaviours (Volkow and Swanson, 2003). One of the other alarming points is the large increase rates of ADHD diagnosis and prescription of medications over the last years which do not represent the rates of true prevalence of the disorder, likely caused by a lack of consistency of strict diagnostic criteria (Polanczyk et al., 2014). This has pointed out the fact that the drug may be inadequately administered to non-ADHD children.

Though ADHD occurs mainly during childhood and adolescence (Adelman, 2001, Hurtig et al., 2007, Wilens and Spencer, 2010) it can persist throughout the entire adult lifespan in a widely ranged proportion of patients (ten to sixty percent), depending on the different cohorts, if previously diagnosed with childhood ADHD (Kessler et al., 2005, Gentile et al., 2006, Pehlivanidis, 2012). Evidence for long-term side effects of psychostimulants is limited. Exposure to psychostimulant drugs on a developing brain may not be without risk, knowing that previous exposure with drugs that have abuse potential can later induce drug-taking and drug-seeking behaviours (Bartoletti et al., 1987, Gubellini et al., 1990, Gaiardi et al., 1991, Shippenberg et al., 1996).

Adolescent exposure to MPH may be associated with altered emotional responses and anxiety-like behaviour in adulthood (Bolanos et al., 2003), increased impulsiveness (Pardey et al., 2012), impaired learning (Rowan et al., 2015) and impaired reproductive axis (Fazelipour et al., 2012). On the positive side, other studies found that chronic MPH can have persistent beneficial effect on prefrontal cortex plasticity and cognition (Burgos et al., 2015). Though childhood or adolescent exposure to MPH to rats at moderate (Brandon et al., 2001, Crawford et al., 2007, Crawford et al., 2011) to high

doses (Achat-Mendes et al., 2003, Halladay et al., 2009) may enhance response to drug of abuse, other studies have consistently demonstrated that low doses can induce an attenuated cocaine-induced conditioned place preference, a measure of the incentive properties of a given drug (Carlezon et al., 2003, Augustyniak et al., 2006, Brenhouse et al., 2009). In contrast, a recent study on ADHD genetic model (spontaneous hypertensive rats) suggests that initiation of methylphenidate treatment in adolescence may increase cocaine abuse risk (Jordan et al., 2014).

Chronic methylphenidate administration may cause persistent alterations in the electrical characteristics of midbrain monoaminergic neurons, potentially causing changes in behavioural functions related to emotion, drug addiction or even cognition. Interestingly, only a few studies have attempted to examine in parallel putative changes in electrophysiological activity and alteration in behavioural function. Two electrophysiological studies reported persistent effects, but in different directions, on dopamine neuronal activity of low doses of MPH (1-2 mg/kg) administered subchronically and intraperitoneally to adolescent rats (1-2 mg/day, 1-3 weeks), and no difference in dopamine autoreceptor sensitivity (Brandon and Steiner, 2003, Shen and Choong, 2006). To our knowledge, no studies have investigated the effect of a juvenile chronic MPH treatment on serotonin neurotransmission. This would be of particular interest as some behavioural studies have shown that chronic juvenile methylphenidate administration at moderate (Bolanos et al., 2003; Carlezon et al., 2003) or high dose (Brookshire and Jones, 2012) can induce depressive-like behaviour and increase sensitivity to stress (Bolanos et al., 2003, Brookshire and Jones, 2012), which may be more specifically related to deficits in serotonin neurotransmission.

The large discrepancy of results in literature may be attributed to differences in doses, length of treatment and washout period, as well as route of administration. There are also disputes about the validity of the doses administered, whether within or over the therapeutic range. Most studies have examined the effects of systemic psychostimulant administration, which is not a reflection of the clinical situation where the drug is usually administered orally. Though methylphenidate has a relatively short half-life (3-4 hr) and low bioavailability (20-40%), it is more slowly absorbed if orally administered,

particularly in drinking water (Wargin et al., 1983, Thanos et al., 2015). Therefore, the total exposure time to the drug may be fundamentally different whether the drug is administered orally or systemically. Oral administration, particularly in drinking water during the dark period should help to maintain plasma concentrations for a longer period, and is therefore more clinically relevant, mimicking to some extents extended release formulations (Thanos et al., 2015). This may impact on the long-term effect of the drug on the different neurotransmitter systems.

In the present study, we have compared the possible long-term consequences of a chronic oral methylphenidate administration on growth, serotonin and dopamine neurotransmission in two age groups of animals: early adolescents (Post Natal Day 28) and late adolescents (PND 42). We have also attempted to characterise a possible link between putative changes in behaviours and electrical characteristics of serotonin and dopamine neurons and dopamine autoreceptor sensitivity. To this end we have combined our electrophysiological investigation with assessments of the motor response to D-amphetamine and of the sucrose preference test, which can assess putative anhedonic behaviour. In addition, in some of our experiments, we have also compared administering MPH as oral daily administration (twice a day, diluted in a sucrose solution) to continuous overnight administration in drinking water. These two procedures should, to some extent, mimic immediate and extended release conditions.

Material and Methods

A- Subjects and groups

Male Sprague-Dawley rats were purchased from Charles River, UK. Animals were housed in groups of 2-4 per cage, maintained at 20-22°C with humidity rates above 40% under a 12:12 L/D cycle with lights ON at 07h00. Food and water were provided *ad libitum*. Animals were allowed a 3-day acclimatisation period after delivery. All experiments were performed during the light phase and with permission from the UK

Home Office and De Montfort University Ethics Committee. During treatment animals were weighted every other day and concentration of MPH in the sucrose solution or drinking water for oral administration was readjusted in order to administer a consistent dose of 5 mg/kg/day per animal. For administration of MPH in the drinking water the daily amount of fluid consumed per animals was also measured in order to evaluate the adequate concentration of MPH in the drinking bottle.

All animals were kept 2-3 per cage and split into the following groups.

-Naïve animals, kept in standard laboratory conditions for the same period as the treated ones (n=22).

-Early adolescent (weight 70-80 g, Post Natal Day (PND) 28, n=22) or late adolescent (150-180 g, PND 42, n=24) animals which were administered MPH (5 mg/kg/day) in a 2 ml/kg 10% (w/v) sucrose solution, twice a day (2x2.5 mg/kg, 0.5 ml volume intake), for 15 consecutive days, followed by a washout period.

-Early adolescent (n=8) or late adolescent animals (n=7) treated with 5 mg/kg/day of MPH incorporated in the drinking water during the dark phase (removed/replaced by water-containing bottles at the beginning of the light phase). Animals of this group were kept 2 per cage and had very similar weight (+/- 5%) within cage. It was also established from preliminary experiments that animals drank very similar amount of water in proportion to their weight, with very little inter-individual variations. In addition, tested and control animals drank the same amount of fluid per day.

Oral administration of 5 mg/kg of MPH by these two methods should not produce plasma peak of MPH concentration higher than the clinical range defined as 8-40 ng/ml (Berridge et al., 2006, Thanos et al., 2015).

Treatments lasted 15 days, followed by a 28-35-day washout period. We paid attention to the fact that animals from all groups, including naïve animals, should have relatively similar weight/age, ranging from 360-460 grams, when tested for electrophysiology/behaviour at a time they were young adults (PND>65). For this reason, late adolescent-treated animals have generally been investigated after a shorter washout period (average 10 days) than the early adolescent-treated rats to keep them

with a similar weight at the time of our electrophysiological and behavioural investigations. However, we also studied a group of late adolescent-treated animals (n=8) with a longer washout (2-3 weeks) and found that the length of the washout period had no impact on electrophysiological and behavioural parameters in these pre-adult-treated animals.

B- Sucrose preference test

After at least 10 days following the last MPH or sucrose administration during early or late adolescence, some animals (n=24) were subjected to a sucrose preference test, adapted from previous published protocols (Overstreet, 2012, Mateus-Pinheiro et al., 2014, Li et al., 2015, Mileva and Bielajew, 2015, Tang et al., 2015). Briefly, rats were tested for sucrose preference over a four-day period using a two-bottle choice test. Animals had *ad libitum* access to food and water throughout the experiment. On the first day, rats were housed singly and accustomed to drinking from two water bottles. On the following three days, rats were trained on the sucrose preference test, in which one out of the two water bottles was replaced with a bottle containing a 2% sucrose solution (w/v). Rats were allowed to drink freely from both bottles during the 12-hour nocturnal phase. At the beginning of the light phase, both bottles were replaced by bottles containing water only. The bottles were weighed and refilled each day at the same time in the morning. The positions of the bottles were switched daily to avoid position preferences which have been observed in mice (Bachmanov et al., 2002). Sucrose preference was determined as the quotient of sucrose consumption to the total liquid intake over a definite period (Day 1, 2, 3, 4 or average Day 2-4), consisting of both water and sucrose intakes. A sucrose preference score lower than 65% was considered a depressive-like phenotype (Willner, 1997, Strekalova et al., 2004, Briones et al., 2012a, Couch et al., 2013).

C- D-amphetamine-induced locomotor activity

At least 20 days following the last MPH administration, some rats (n=21) were housed individually for the behavioural experiments. All drugs were dissolved into saline. Animals received a single intraperitoneal injection of either: 0.8 ml/kg of saline, or 1 mg/kg of D-amphetamine in saline. Animals were then scored for behavioural parameters during 15-minute time periods and up to a total of 60 minutes following the injection. Counting of well-defined behavioural traits such as rearing, scratching, grooming, jumping, running, climbing, catalepsy and stereotypical movements were done manually. Drugs or saline were injected approximately 30 min after the rat was put in the individual testing cage and the observation period started 2 minutes after administration. Saline administration was carried out in a group of 4 animals from both naïve and early-adolescent treated animals. At the end of the assessment, animals return to their original cage.

D- *In vivo* extracellular single unit electrophysiology
1-General recording procedures:

Animals were anaesthetised with chloral hydrate (400 mg/kg, with additional doses administered if necessary), secured to a stereotaxic frame and maintained at 36–37 °C with a heating pad. An incision was made across the top of the head and the edges of the skin drawn back to reveal the cranium. Bregma was marked and a hole was drilled through the bone at the coordinates of the region of interest to the atlas of Paxinos and Watson (1997).

Electrodes were manufactured in house from borosilicate capillaries (1.5 mm, Harvard Apparatus Ltd., UK) pulled on a PP- 830 electrode puller (Narishige, Japan) and filled by hand with an electrolyte solution (in mM: NaCl 147, KCl 4, adjusted to pH 6). The tip of the electrode was broken down under a microscope to an external diameter of 1–1.5 µm. Typical resistance was in the range 4–8 MΩ. Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifier (Digitimer, UK). Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope, and a 1401 interface connected to a computer running Spike 2 (CED, Cambridge, UK) for data capture and analysis. Descent of the electrode was carried out using a hydraulic micromanipulator (Narishige). Typically, a minimum of 7 cells were recorded per

animal (particularly for measuring average electrophysiological parameters). No more than 10 electrode descents were performed per animal.

2- Serotonin neurons

Only putative serotonin neurons with a large and long-lasting triphasic extracellular potential (1-2 ms), a low but regular firing activity (1-40 spikes per 10 seconds) were recorded (Vandermaelen and Aghajanian, 1983, Urbain et al., 2006, Oosterhof et al., 2015). To further confirm the specific recording of serotonergic neurons, systemic exposure to 8-OH-DPAT (5-Hydroxy-*N,N*-dipropyl-2-aminotetralin) was performed at the end of some recordings, which induced neuronal silencing that could be reversed by WAY-100135 administration. Bursting of DRN serotonin neurons was calculated with the following criteria: at least two bursts occurring within 20 ms or less and followed by a silence period of at least 40 ms, adapted from previous electrophysiological investigations (Labonte et al., 2012; Manta, Dong, Debonnel, & Blier, 2009; Rouchet et al., 2008). The number of putative DRN serotonin neurons per track was recorded by recording the total number of active serotonin neurons encountered during one electrode descent within the DRN, at the following coordinates: anteroposterior -7.5 to -8 mm to Bregma, lateral 0 mm, dorsoventral 5-7 mm below cortical surface.

3- Dopamine neurons

Midbrain dopamine neurons were identified according to criteria summarised by Ungless and Grace (Ungless and Grace, 2012). Presumed dopaminergic neurons displayed typical characteristics such as: a triphasic action potential lasting 2.5-3.5 ms often with a notch in the rising phase, a prominent negative compound and a time greater than 1 ms from the start of the depolarisation to the end of the repolarisation, a relatively low firing activity between 5-90 spikes/10 s with occasional bursting pattern. A burst activity in such neurons is defined as two spikes occurring at an interval of 80 ms or less, followed by a silence period of at least 160 ms. Coordinates for the ventral tegmental area were: anteroposterior -4.5 to -5.7 mm to Bregma, lateral 0.3-1.2 mm, 7.2-9.5 mm below cortical surface.

E- Data analysis

Results are expressed as the mean±standard error of the mean (SEM). Statistical comparisons were carried out using either, as appropriate, one-way analysis of variance (ANOVA) followed by Newman-Keuls tests or two-way ANOVA followed by Bonferroni post hoc analysis. Statistical significance was considered if $p<0.05$. For behavioural experiments, the sum of each behavioural trait occurring during 15-minute interval periods was considered. The mean basal firing activity was evaluated after the neuron had attained a stable firing rate, generally after at least 5 min of recording. In case of drug administration: pre-drug values of firing rate were obtained by averaging the firing rate over a period of at least 2 min immediately prior to the intravenous administration of the drug, while post-drug values were obtained by averaging the firing over a period of 4 min following drug administration. Proportions of a specific type of response in two different groups of animals were also compared using the Fisher's exact or Chi-squared test (comparing proportions of responses and no response/opposite responses in two or more groups).

Results

A- Adolescent treatment with methylphenidate does not induce growth deficits

The impact of a chronic methylphenidate treatment during adolescence on growth parameters was investigated. We noted with interest that MPH, administered orally at 5 mg/kg/day and for 15 days (in drinking water or via a sucrose solution), did not induce any body weight gain deficiency during the treatment period (not shown). After the washout period, no differences were observed between MPH-treated animals and vehicle-treated animals. In another study, we found the same absence of effect after 15 days of intraperitoneal MPH administration (4 mg/kg/day), though more individual variations were noted (Di Miceli and Gronier, 2014).

B- Sucrose preference test

This test was performed in early (8 MPH treated and 4 controls) and late adolescent-treated (8 MPH treated and 4 controls) animals which were administered sucrose only or MPH in a sucrose solution (10 %) twice daily (2.5 mg/kg x 2). At the end of the washout period (at least 8 days after the last administration), animals were assessed for the sucrose preference-test. Using a protocol that evaluates sucrose preference in basal condition (non-water-deprived, see Briones et al., 2012b), we found that animals successfully learnt to discriminate water from sucrose. Indeed, the transition from the first day to the second day is marked by an increase in liquid consumption (Fig. 1A) found in 11 out of 12 animals (92%) from the early-adolescence group and 12 out of 12 from the late-adolescence group. Interestingly, after this initial increase in sucrose consumption, animals tend to decrease their sucrose consumption on the third day to finally maintain this level of consumption the following days (Fig. 1A). MPH-treated animals and sucrose only-treated animals did not differ in their sucrose preferences (Fig. 1B-C), in both early and late adolescence-treated groups. The low scores, observed in 25% of early-adolescence-treated animals, were not due to individual decreases in total volume intake, as both control and tested groups displayed similar liquid intake patterns (Fig. 1A). We use the 65% threshold to discriminate anhedonic-like behaviours (Willner, 1997, Strekalova et al., 2004, Briones et al., 2012b, Couch et al., 2013). Therefore MPH treatment did not induce any long-term anhedonia traits in rats, whether early adolescence or late adolescence-treated. This experiment was reproduced in naive animals (n=10) and the same low frequency of this phenotype was observed (10%, Fig. 1D), indicating that previous sucrose intake (during the chronic MPH administration) has no impact on sucrose preference.

C- D-amphetamine-induced locomotor activity

When D-amphetamine is administered intraperitoneally (>1 mg/kg) to naive adult rats, it induces considerable rearing activities in the first hour following the administration. In general, no rearing events were recorded during the last 15 min before administration of the drug. On the other hand, administration of saline only marginally caused rearing events in both control or tested animals (not shown).

In the present study, we used a low dose of D-amphetamine (1 mg/kg) which induces peak rearing events activity during the first 30 minutes of observation. This was followed by progressive time-dependent decreases of such events, in both control and tested groups (Fig. 2). In both groups of control animals (sucrose-treated, n=8, or naïve rats, n=12) similar changes in rearing activity was observed over the time course of the experiments (average number of rearing events, sucrose/naive: 18/23, 41/35, 39/38, 35/21 for 0-15, 15-30, 30-45 and 45-60 min, respectively). Likewise, animals treated with MPH, either administered in sucrose solution (n=7) or in drinking water (n=8), displayed similar changes in rearing activity following the D-amphetamine challenge (average number of rearing events, sucrose/drinking water: 49/41, 60/47, 43/36, 46/35 for 0-15, 15-30, 30-45 and 45-60 min, respectively). The number of rearing activity was slightly higher in the sucrose group, but this was not statistically significant due to large individual variation ($p < 0.4$, unpaired Student's test, 15-30 min). Data from both control groups and from both treated groups were therefore pooled. Overall, we observed that animals previously treated orally with MPH during early adolescence (5 mg/kg/day) displayed significantly more frequent rearing events during the first 15 minutes of observation (Fig. 2). The two-way ANOVA analysis of average rearing activity shows a significant effect of treatment ($F_{1,132} = 7.99$, $p < 0.005$), which is attributed to an increase in rearing activity in the group of animals pre-treated with MPH during early adolescence for the first 15 min following the D-AMP challenge ($p < 0.05$), compared to control rats. The number of rearing/vertical events tend to remain higher during the 15-30 min periods in the tested groups, but this was not statistically significant, ($p < 0.09$, unpaired Student's t test). On the other hand, the proportion of rats that displayed high rearing activity (arbitrarily defined as > 10 events during each 15-min test period) was significantly higher in the treated group than in the control group during the 0-15 min period (12/15 versus 8/20, $p < 0.04$, Fisher exact test) and during the 15-30 min period (11/15 versus 8/20, $p < 0.04$).

Interestingly, this increase in rearing activity observed during the first 15 min following the administration of D-AMP was not observed in a group of 6 rats that were previously orally treated with MPH during post adolescence (following at least a 10-day washout period). These rats show similar behavioural activation than the control rats and

significantly lower rearing activity than the early adolescence MPH-treated animals (Fig. 2).

All of the other behavioural events (scratching, grooming, jumping, running, climbing, catalepsy, stereotypical movements), which were occasionally triggered by D-AMP, did not occur more frequently in any of the animal subgroups, during any interval times of our monitoring.

D- Adolescent MPH leads to long-term adaptations in serotonin neurons in adulthood

Three to five days following the sucrose preference test, serotonin neurons from the dorsal raphe nucleus (DRN) were examined *in vivo* in some animals (n=23). Typically, at least 7 neurons were studied per animal. MPH, administered orally at 2.5 mg/kg twice a day for 15 consecutive days during early adolescence (n=12), did not change the firing activity of DRN serotonin neurons compared to rats treated with sucrose only during early adolescence (Table 1). However, it significantly increased ($p < 0.007$, Student's t-test) burst discharges in these neurons by 4-fold (Fig. 3B). In this group, 26% of the neurons tested (13/50) displayed bursting activities whereas only 12% did so in respective control group (8/65, $p < 0.05$, Fisher test). In rats administered MPH in late adolescence (n=11), no significant changes of the electrophysiological properties of 5-HT neurons were found at adulthood, when compared to rats treated with sucrose only during late adolescence (Table 1). Figure 3A-C shows that burst activity, but not firing activity nor population activity, of serotonin neurons is significantly higher in the early adolescence MPH-treated group than in all control animals (early and late adolescent sucrose-treated animals grouped together).

Interestingly, in the early adolescence MPH-treated animals (n=7), we found a negative correlation between sucrose preference results and average burst activity of 5-HT neurons (Fig. 3D), but not with the other firing parameters (not shown). In control animals (sucrose solution only) and in late adolescence MPH-treated animals, we did not observe such correlation, probably due to the fact that average bursting activities per animal were lower.

E- Adolescent MPH leads to long-term adaptations in midbrain dopamine neurons in adulthood

Control animals (n=4) that were orally administered the sucrose solution during adolescence displayed similar firing and burst activities of ventral tegmental area (VTA) dopaminergic neurons than untreated control animals (n=5; Table 1). VTA dopamine neuron firing and burst activities from both groups of animals exposed to MPH during early adolescence (in sucrose solution, n=4, or in drinking water, n=6) did not differ significantly (Table 1). Firing activities tend to be higher in the two groups of rats treated during adolescence, compared to their respective control groups but the differences were not statistically significant. On the other hand, previous exposure to MPH during early adolescence consistently increased baseline burst activities in each subgroup of orally treated rats compared to respective controls; this effect was significant in the group of rats administered MPH in drinking water (unpaired t-test, $p<0.03$).

Dopamine cells from late adolescence MPH-treated animals had also similar firing and burst activity whether MPH was administered in sucrose solution (n=8) or drinking water (n=4) and have very similar values than rats administered sucrose during late adolescence (n=9) or untreated control animals (n=5) (Table 1). In both groups of animals exposed to MPH during late adolescence, burst activity of VTA dopamine neurons was lower than in the corresponding early adolescent groups exposed to MPH, but this was significant only in the group where MPH was administered in drinking water ($p<0.03$, unpaired Student t test, Table 1).

We then pooled the animals into three groups: rats previously treated orally with MPH (whether in sucrose solution or in drinking water) during early adolescence, rats previously treated orally with MPH during late adolescence and control rats (whether sucrose-treated or age and weight matched naive). We found that electrophysiological characteristics of VTA dopamine neurons were affected by chronic oral MPH treatment during early adolescence. Both firing and burst activities were significantly higher in the group of rats previously exposed with MPH during early adolescence, compared to controls and to rats treated during late adolescence (Fig 4). We also found that

population activity (expressed as the number of spontaneously discharging dopamine neurons encountered per electrode descent) was also significantly increased in rats treated with MPH orally as early adolescents, compared to the other groups (Fig. 4).

F- Adolescent MPH exposure induces partial dopamine D₂ receptor desensitisation in adulthood

In these experiments, we combined data from animals that were orally administered MPH in sucrose solution or in drinking water, as well as from all control animals (subsequent analysis of our data did not show any differences in response to dopamine receptor agonists between different subgroups). Using a wide range of apomorphine (a D₂ receptor agonist) doses intravenously administered, with progressive 10 µg/kg increments and up to a cumulative dose of 50 µg/kg (or 70 µg/kg in some animals), animals previously exposed to MPH during early adolescence displayed a significant shift in their sensitivity to the D₂ agonist, as seen in Fig. 5A-B. Analysis indicates a significant dose ($F_{5,68}=12$, $p<0.0001$) and treatment effect ($F_{1,68}=24$; $p<0.0001$) with no significant interaction ($F_{1,68}=1.8$; $p<0.13$). Treatment effect was attributable to a significantly higher firing rate in MPH-treated rats at the dose of 10, 20 and 30 µg/kg of apomorphine (Fig. 5A). Equivalent results were obtained when % of firing decreases from baseline were considered (Fig. 5B). Figure 5A-B also shows that the depressing effect of apomorphine on the firing rate was reversed by the D₂ antagonist eticlopride. Figure 5C shows representative firing rate histograms and burst train of a VTA dopamine neuron from an early adolescence MPH-treated rat during administration of cumulative doses of apomorphine, which displayed such partial insensitivity to the agonist.

This prompted us to carry out another study on dopamine D₂ autoreceptor sensitivity to compare MPH-treated animals in early and late adolescence, using quinpirole (a more selective D₂ receptor agonist). Intravenous administration of quinpirole (20 µg/kg) led to strong and reversible (following eticlopride injection) firing rate reductions of VTA dopamine neurons (Fig. 6A-B). Dopamine neurons from animals exposed to MPH during early adolescence displayed lower sensitivities (assessed as the percentage of decrease in the firing rate) to the dopamine D₂ receptor agonist (78, 45 and 75% for controls, early and late adolescent MPH-treated groups, respectively, $F_{2,23}=3.64$,

p<0.04, Fig. 6B). As indicated in the figure, there is a significantly lower effect of quinpirole on dopamine neurons from rats initially treated with MPH during early adolescence compared to animals treated lately and control rats (p<0.05, Newman-Keul test). The proportion of neurons completely or nearly completely silenced (firing<5 spikes/10 s) by quinpirole was significantly different in the three subgroups (6/11, 0/7, 4/7 neurons from the control, early adolescent-treated and late adolescent-treated groups, respectively; p<0.04, Xi square test).

Discussion

Our results, showing that adolescent exposure to MPH does not induce growth velocity deficits corroborate previous observations (Pizzi et al., 1987, Achat-Mendes et al., 2003, Spencer et al., 2006).

In adult rodents, adolescent exposure to high (Brookshire and Jones, 2012, Motaghinejad et al., 2015) or therapeutic (Carlezon et al., 2003, Bolanos et al., 2003) dosing of MPH has been associated with depressive-like effects, at least based on increased immobility in the forced swim test in rats when tested during adulthood; though this was not always observed (van der Marel et al., 2015). Our study supports the assumption that early or late adolescence MPH administrations did not induce more anhedonic-like behaviour in adulthood (Fig. 1) than what is generally found in the background population. Interestingly, we showed that some control animals (n=2) and one early adolescence MPH treated animal display sucrose preferences below 65%. This stands in contrast to other investigations using the same protocol and strain of animals showing sucrose preference above 70% on all control rats (Wang et al., 2014, Boyko et al., 2015). One previous study found that adolescent exposure to MPH (2 mg/kg, i.p., twice a day for 16 days) significantly decreases the sucrose preference of adult rats for solutions ranging from 0.1 to 0.5% but not with a 1% sucrose concentration (Bolanos et al., 2003), while we used a 2% concentration (Fig. 1). On the other hand, other studies have reported unaltered sucrose preference (using 0.125% sucrose solution) in adulthood in singly-housed rats after adolescent chronic MPH administration (2-5

mg/kg, once daily, i.p., 10 days), with sucrose preferences ranging between 59 and 72% (Crawford et al., 2013), in consistence with our own observations.

When dorsal raphe nucleus serotonin neurons were recorded during adulthood, we did not see any change in the firing activity or in the total number of active neurons per track following chronic adolescent (early or late) MPH treatment (Table 1, Fig. 3). However, we found that an early adolescence exposure to MPH leads to increased burst activities of DRN serotonin neurons (Fig. 3B). In a similar way to dopamine neurons (Overton and Clark, 1997), bursting activity of DRN serotonin neurons is known to result in a greater efficiency of serotonin release in serotonin projecting region (Hajos and Sharp, 1996, Gartside et al., 2000, Hurtig et al., 2007, Hajos et al., 2007). Some serotonin neurons in the DRN may have adapted their firing mode following chronic MPH, by switching from single spike activities to bursting activities, without increasing their firing rates (Jennings, 2013), an effect that persists in time. Baseline burst levels in the control group, around 5%, was similar to previous findings (Manta et al., 2009, Schweimer and Ungless, 2010). The mechanism triggering burst activity in serotonin neurons is still poorly understood. Similarly to dopamine neurons, previous studies have found that SK channels (small conductance calcium-activated potassium channels) negatively control the firing pattern of 5-HT neurons (Rouchet et al., 2008). It is not known whether MPH administration has caused a down regulation of these ion channels. In addition, it will be necessary to examine other mechanisms that may also contribute to promote burst activity in serotonin neurons, such as the glutamatergic input via NMDA receptors, as observed in several other neuronal systems. One of the physiological significances of burst activity from DRN serotonin neurons may be to mediate the anticipation of reward, but not punishment. Indeed, during the anticipation period of a sucrose reward, mice displayed strong burst activity of DRN serotonin neurons, an effect not seen during the rewarding event itself (Cohen et al., 2015, Li et al., 2016).

Interestingly, in our experiments, a strong negative linear correlation ($r^2 = 0.755$) between sucrose preference and the burst activity of DRN serotonin neurons was observed in early adolescence MPH-treated animals (Fig. 3D). This may indicate a possible direct relationship between depression-like traits and the electrical activities of

serotonin neurons. Anhedonia can reflect a state of stress which can disturb the reward system and cause these changes in firing pattern in some serotonin neurons which are “reward-sensitive”. In addition, it should be emphasized that serotonergic neurons in the DRN are not homogenous, with some groups being electrically responsive to stressor events (Crawford et al., 2010). More studies will be needed to characterise the exact relationship between depression-like traits in rodents and serotonin neuron electrical activities.

We found that early but not late adolescence exposure to MPH induced a significant increase in sensitivity to D-amphetamine, assessed by an immediate but relatively short-lasting increased number of rearing events induced by D-amphetamine (1 mg/kg, intraperitoneally). This was observed in adult animals previously exposed to MPH during early adolescence (Fig. 2), but not in adult rats exposed to MPH during late adolescence. Such effect may underlie plastic mechanisms that occurs following stimulant exposure during adolescence (Simchon Tenenbaum et al., 2015, Quansah et al., 2017). Though not totally understood, rearing activity can be considered as a good (but certainly not the only one) index of a motor response following drug administration (al-Khatib et al., 1995, El Yacoubi et al., 2000). Various agents, particularly dopamine releasing drugs, drive increases in both ambulation and rearing (Lever et al., 2006) which seems most likely related to striatal activities. This result stands in contrast to a previous study, where the authors found no relationship between adolescent exposure to MPH and sensitisation to methamphetamine in later life (Kuczenski and Segal, 2002). However, our study only focussed on rearing behaviour, which was only found to be increased in the first 15 minutes following the D-amphetamine administration. Furthermore, there was a difference in the dose administered and the route of administration of MPH during adolescence (gavage in Kuczenski study, which can induce stress and potentially confound experimental measurements). On the other hand, evidence of locomotor cross sensitisation exists in literature. Brandon *et al* (2001), using adolescent male Sprague-Dawley (SD) rats, demonstrated that pre-treatment with MPH may have a sensitising effect towards use of cocaine in adulthood, while also increasing self-administration of cocaine (Brandon et al., 2001). Other studies have also demonstrated locomotor sensitisation or cross-sensitisation, following a chronic i.p.

MPH administration, whether in adult or adolescent animals (Yang et al., 2003, Yang et al., 2007, Yang et al., 2011). Nevertheless, our findings indicate a striking difference between early and late adolescent treated animals, indicating that utilisation of MPH during early adolescence may sensitise and/or predispose some sensitive subjects to later use of psychostimulant drugs, because important neural changes take place within the dopaminergic circuitry during this stage of development. However, in our study the effects observed are relatively mild and short-lasting, with inter-individual variation, indicating that, in clinical situation, only a fraction of subjects could be particularly vulnerable. In addition, these experiments were performed in healthy animals which prevent us to predict that ADHD patients will be equally sensitive. To note, however, it is widely admitted that psychostimulant drugs are sometimes not appropriately prescribed (Polanczyk et al., 2014).

This behavioural observation stands in concordance with our electrophysiological data showing persistent alteration of dopamine electrophysiological activity caused by early adolescent MPH treatment, as shown by an increase in firing, burst and population activities (Fig. 4). Previous studies examining the effects of systemic treatment with MPH in adolescence found different results. According to Brandon (Brandon et al., 2001) a short withdrawal following MPH exposure during adolescence (1 week, 2 mg/kg i.p.) tends to increase both firing and burst activity, while long withdrawal (approximately the same period as in our study) significantly depressed firing activity (Brandon et al., 2003). There are differences in dose, route of administration, as well as period of treatment with our study. Such treatment may be too short and therefore not adequate to induce the persistent increase in dopamine neuronal activity that we have found. The i.p. administration may only induce a short-lasting peak of MPH in plasma, reducing the time by which the body is exposed to the drug. Oral administration is more likely to mimic the clinical situation, even if 5 mg/kg of oral MPH may look like a relatively high dose (though likely within therapeutic range, see Berridge et al., 2006 and Thanos et al., 2015), it was given either continuously (overnight drinking water) or in two separate half doses (2.5 mg/kg), which likely prevent the occurrence of short lasting peak level of plasma MPH (Berridge et al., 2006, Thanos et al., 2015). It is also important to note that the use of the two slightly different procedures of oral

administration (single dose intake or continuous administration) produced similar effects in our study. Other authors, Shen and Choong (2006) reported an increase in population activity after a short withdrawal which faded after long withdrawal, where a decrease in population activity was found in most animals (Shen and Choong, 2006). The authors attributed this fall in population activity to depolarisation-inactivation process, as population activity returned to normal following the administration of the dopamine D₂ receptor agonist apomorphine. This effect was also observed by the same authors with other psychostimulants, such as amphetamine, cocaine, ethanol and nicotine (Choong and Shen, 2004). Depolarisation-inactivation is likely a consequence of excessive cell excitability. The change in excitability observed in our study obviously does not reach the extent of inducing a state of depolarisation-inactivation, like in Shen and Choong studies. Such changes in excitability may be caused by augmented glutamatergic neurotransmission onto VTA DA neurons, as has been suggested by others (Vanderschuren and Kalivas, 2000, Wanchoo et al., 2009, dela Pena et al., 2015, Creed et al., 2016). In partial agreement with our study, other authors have also found that adolescent amphetamine exposure, followed by a washout period, increased both dopamine and serotonin neuronal excitability, which was, however, only associated with increase in firing activity (Labonte et al., 2012).

Interestingly, our data also demonstrated partial desensitisation of dopamine D₂ autoreceptors located on VTA dopamine neurons. When apomorphine, a potent, but not selective dopamine D₂ receptor agonist, was given intravenously to adult rats previously exposed to chronic MPH during early adolescence, it produced a lower firing rate reduction than in control animals, proving a partial desensitisation of dopamine D₂ autoreceptors (Fig. 5). We repeated this observation with another more selective dopamine agonist (quinpirole) and compared the sensitivity of dopamine neurons in the early and late adolescent MPH-treated groups. We found a similar reduction of dopamine D₂ receptor sensitivities, associated with a significantly smaller proportion of neurons that were completely inactivated by the agonist only in the early adolescent-treated group (Fig. 6). Interestingly, in midbrain dopamine D₂ receptor deficient mice (induced by conditional dopamine D₂ receptor knockout), cocaine induced a stronger locomotor activity than in wild-type animals (Bello et al., 2011). One can wonder

whether the partial desensitisation of the VTA dopamine D₂ auto-receptors observed in our study can be associated to the greater locomotor response to D-amphetamine, at least in the initial phase of the challenge. A partial reduction of autoreceptor sensitivity will attenuate negative feedback mechanism on dopamine cells, preventing the neuron to reduce its electrical activity following VTA dopamine overflow caused by the releasing agent, leading to more dopamine release in terminal area. We therefore hypothesised that chronic MPH treatment during early adolescence may induce persistent changes in dopamine D₂ receptor sensitivity. As discussed before, whether this can then translate into increased sensitivities to addictive drugs is still an open question. Interestingly, higher electrical activity of dopamine neurons and lower sensitivity to D₂ agonist has also been associated to enhanced vulnerability to cocaine self-administration (Marinelli and White, 2000). In ADHD patients, dopamine neurotransmission is disturbed, and the persistent changes we observed may not have the same effect than in healthy individuals. For instance, dopamine D₂ receptor density/sensitivity may be increased in ADHD (Cho et al., 2014), as a consequence of increased levels of dopamine transporter and reduced synaptic dopamine concentration (Fusar-Poli et al., 2012). Our study may indicate that such disturbance can be amended after chronic MPH administration.

Interestingly, in rodents, diet-induced obesity caused lower VTA dopamine-D₂ autoreceptor sensitivity (Koyama et al., 2014) and higher D-amphetamine induced activity (Naneix et al., 2017). It would be particularly important to examine whether this partial desensitisation observed in our study is only observable on the dopamine autoreceptors found in the VTA, or if such a desensitisation can also affect another population of dopamine D₂ receptors, such as the postsynaptic D₂ autoreceptors found in striatal medium spiny neurons.

In conclusion, we have demonstrated that adolescent exposure to MPH, followed by drug withdrawal, does not induce delayed growth or anhedonia-like behaviour. However, exposure to chronic MPH during early adolescence affects the electrical functions of DRN serotonin and VTA dopamine neurons. Dopaminergic neurons display higher level of electrical activity which may be associated to both a partial dopamine D₂ autoreceptor desensitisation. It also appeared that early adolescence oral

exposure to MPH can increase sensitisation to later D-amphetamine administration in adulthood. To our knowledge, we describe here for the first time the differences between early and late adolescence exposure to methylphenidate. Moreover, our protocol was chosen to best mimic clinical situations, using similar doses, routes of administration and length of treatment. In this regard, it is worth mentioning that in our study, single oral dose intake or continuous administration, comparable to some extents to immediate and to extended release, caused similar long-term effects. It is of course not possible to claim from our data that MPH treatment can put all patients at risk for drug abuse during later life. It may be on the contrary that these persistent effects help to restore a deficient dopaminergic system and contribute to prevent the reoccurrence of the ADHD symptoms in adulthood. On the other hand, if administered to a healthy child who has inappropriately been diagnosed with ADHD, our data indicates that administering the drug may induce persistent changes on dopamine-dependent behaviours that can have detrimental consequences.

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Legends:

Table 1: Electrophysiological characteristics of dopamine neurons and serotonergic neurons according to periods of treatment and administration routes.

**p<0.01, vs. corresponding vehicle-treated animals; *p<0.03, vs. naïve or corresponding vehicle-treated animals, #p<0.03, vs. corresponding early adolescence-treated animals, unpaired Student’s t-test; n indicates the number of cells recorded from 4-9 rats per subgroup.

Figure 1: Chronic adolescent exposure to methylphenidate and sucrose preference at adulthood.

(A): Control animals and MPH-treated animals from both groups (late and early adolescence) successfully learned to discriminate water from sucrose, assessed by a drastic increase of volume intake from day 1 to day 2, corresponding to the transition between water and sucrose. Average volume intakes (water or sucrose) are not different between control and MPH treated animals in the two groups during the entire experimental protocol. Note that the late adolescence group tends to drink more fluid because the animals were significantly bigger. (B): Animals from both groups (early and late adolescence) showed similar sucrose preference scores (expressed as the quotient of sucrose consumption to the total liquid intake over each day). (C): Average sucrose preference scores over the test period (Day 2-4). Note that 1 animal out of 4 in controls, and 2 treated animals out of 8 from the early adolescence-treated groups

displayed low sucrose preference (score below 65%, dashed line). Late adolescence treated animals show similar profile of sucrose preferences as related controls and as early adolescence treated animals, but with less scattered values. **(D)**: To confirm if any anhedonic-like behaviour was naturally occurring in our batch of animals, 10 naive animals were also scored for sucrose preference at the same age as the adolescent-treated animals. We observed that 10% of all animals (1/10) could be considered as displaying anhedonic-like behaviour (score below 65%).

Figure 2: Early adolescence exposure to methylphenidate induces changes in rearing activity to later D-amphetamine challenge.

During the first 15 minutes of observation following D-amphetamine (1 mg/kg, intraperitoneally) challenges, MPH-treated animals during early adolescence displayed significantly greater rearing events than control animals. On the other hand, adult rats previously treated with MPH during late adolescence did not display any significant increase in rearing activity during the initial part of the challenge.

* $p < 0.03$ vs. vehicle and MPH late adolescence, Student's t-test; n indicates the number of rats tested.

Figure 3: Electrophysiological characteristics of dorsal raphe nucleus serotonin neurons following chronic MPH exposure and correlation to sucrose preference.

(A, B, C): Chronic MPH treatment during early adolescence, but not late adolescence, increased the burst activities of dorsal raphe nucleus (DRN) serotonin neurons at adulthood, without significantly changing the firing rates nor the total number of active neurons per electrode descent. ** $p < 0.01$ vs. controls; Newman-Keuls test after significant one-way ANOVA; n indicates the number of neurons tested **(A, B)** or electrode descents **(C)**, from 8-15 animals per group. **(D)**: In early adolescence MPH-treated animals, but not in the other groups, a strong correlation was found between the bursting activities of DRN neurons and sucrose preference scores, but not with cell firing activities nor the total number of active neurons (not shown), showing that high burst activities are correlated to low sucrose preference scores. r^2 value indicates the correlation coefficient. **(E, G)**: Representative firing examples of two serotonergic

neurons displaying burst activities, each recorded from a MPH-treated animal. The arrows represent the magnified position chosen in **F** and **H**. (**F**, **H**): Burst events, consisting of doublets of spikes in short intervals.

Figure 4: Electrophysiological characteristics of ventral tegmental area dopamine neurons following chronic MPH exposure.

Chronic treatments with MPH (5 mg/kg/day) during late adolescence did not alter firing rate (**A**), burst activities (**B**) or total number of spontaneously active dopaminergic neurons per electrode descent (**C**). However, chronic exposure to MPH during early adolescence significantly increased firing, burst, and population activities of midbrain dopamine neurons measured at adulthood. ** $p < 0.01$ vs. controls and adult-treated; * $p < 0.05$ and ** $p < 0.01$ vs. controls; Newman-Keuls test after significant one-way ANOVA; n indicates the number of neurons tested (**A**, **B**) or electrode descents (**C**), from 10-15 animals per group.

Figure 5: Early adolescent exposure to chronic methylphenidate induces partial desensitisation of dopamine neurons to apomorphine in adulthood.

(**A**): MPH-treated animals during early adolescence displayed significantly lower firing rate reductions following progressive apomorphine challenges, administered in a dose-response manner and with progressive 10 $\mu\text{g/kg}$ increments. * $p < 0.05$ vs. controls, Bonferroni after significant repeated measures two-way ANOVA. Note that eticlopride successfully reversed apomorphine-induced firing rate reductions. (**B**): Compared to controls, animals previously exposed to chronic MPH in early adolescence had significantly lower sensitivities to dopamine D_2 receptor agonism ($F_{1,8} = 12.57$, $p < 0.01$). n indicates the number of neurons/rats tested. * $p < 0.05$ vs controls, Bonferroni after significant repeated measures two-way ANOVA. (**C**): Representative firing example of a dopaminergic neuron from an early adolescence MPH-treated animals displaying burst activities, together with a weak sensitivity to cumulative apomorphine administration.

The two bottom arrows represent the magnified position chosen in **D. (D)**: Burst event, consisting of a triplet of spikes.

Figure 6: Early but not late adolescence exposure to chronic methylphenidate partially induces desensitisation of dopamine neurons to the D₂ agonist quinpirole in adulthood.

(A): Intravenous challenges with quinpirole (20 µg/kg, another D₂ receptor agonist) significantly reduced the firing activities of VTA dopamine neurons in both control and MPH-treated animals ($p < 0.0001$). Eticlopride (0.2 mg/kg) successfully recovered such firing rate reductions. (B): Methylphenidate-treated animals during early adolescence but not during late adolescence presented a lower sensitivity to quinpirole challenges in adulthood. * $p < 0.05$, compared to control and adult-treated rats, Newman-Keul test after significant ANOVA; n indicates the number of neurons/rats tested.

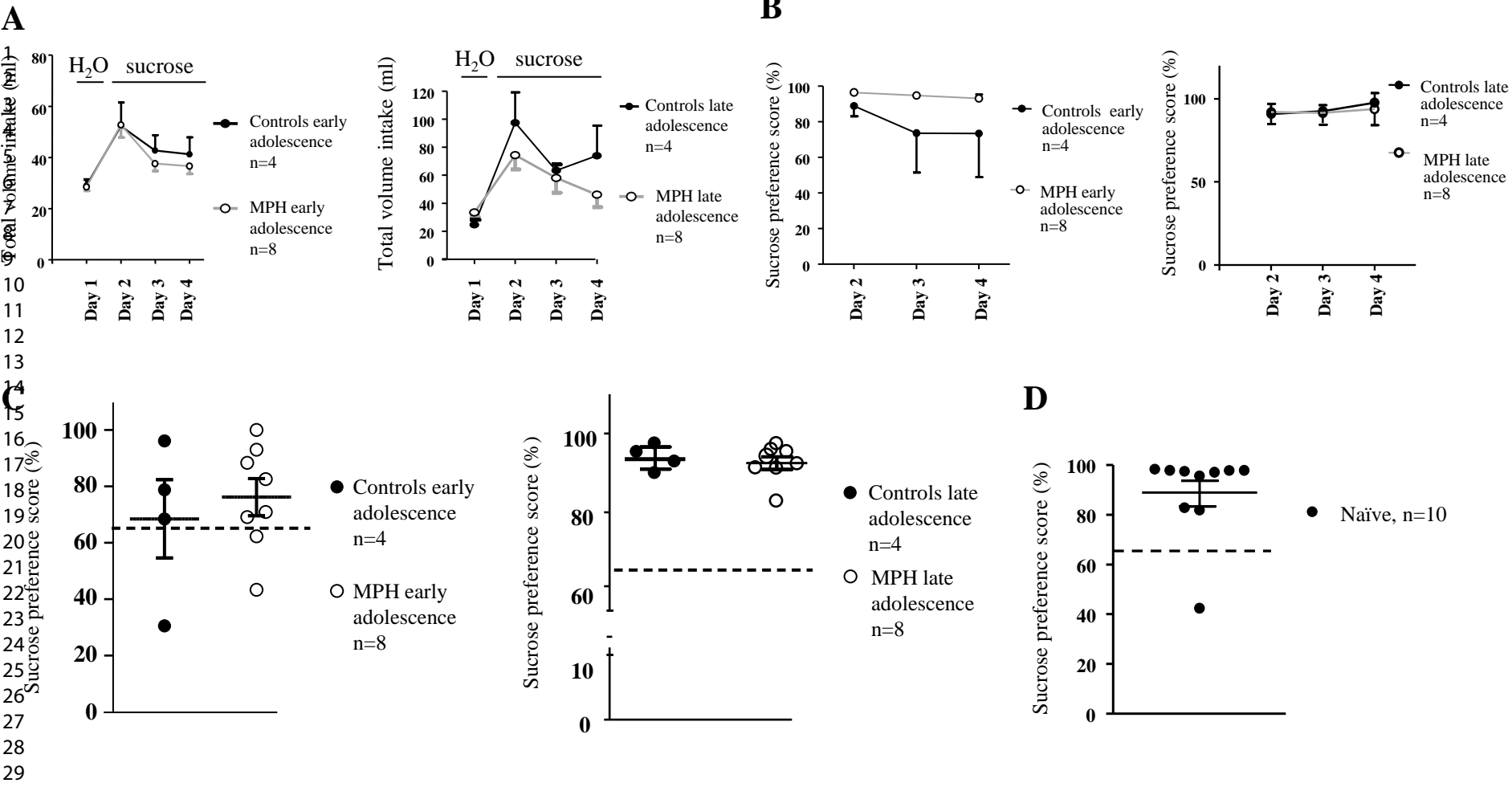


Figure 1: Chronic adolescent exposure to methylphenidate and sucrose preference at adulthood.

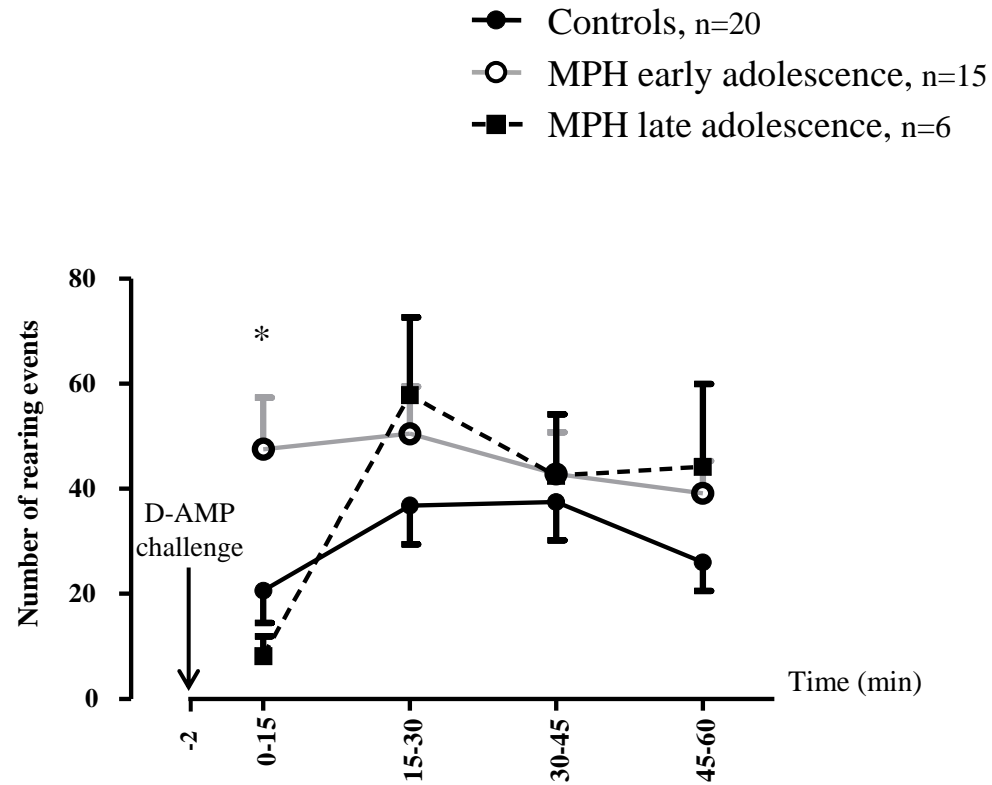
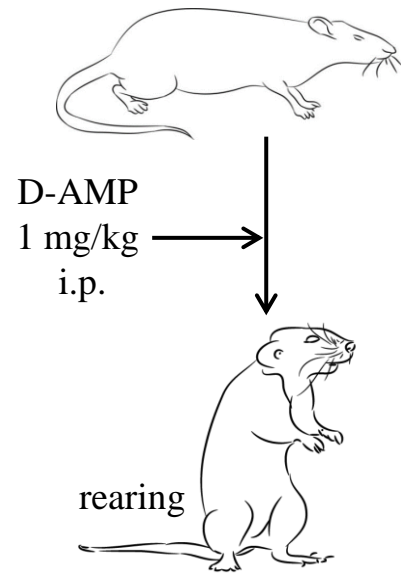


Figure 2: Early adolescence exposure to methylphenidate induces changes in rearing activity to later D-amphetamine challenge.

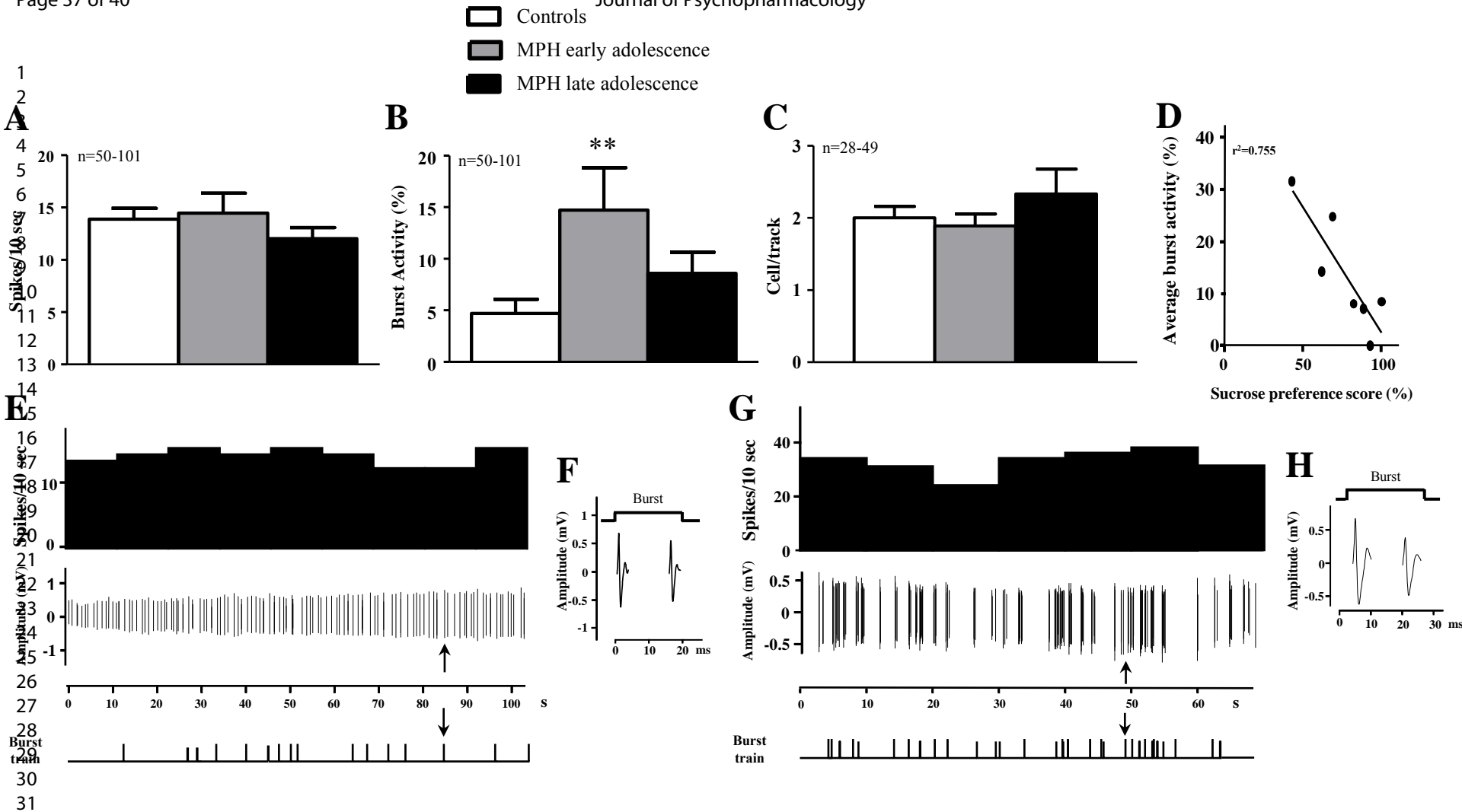


Figure 3: Electrophysiological characteristics of dorsal raphe nucleus serotonin neurons following chronic MPH exposure and correlation to sucrose preference.

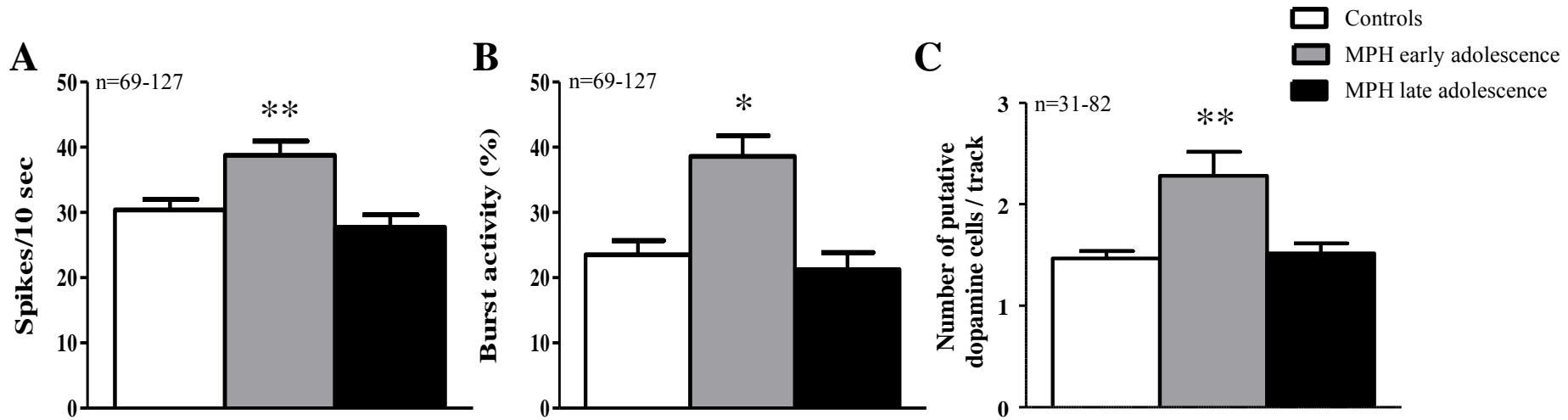


Figure 4: Electrophysiological characteristics of ventral tegmental area dopamine neurons following chronic MPH exposure.

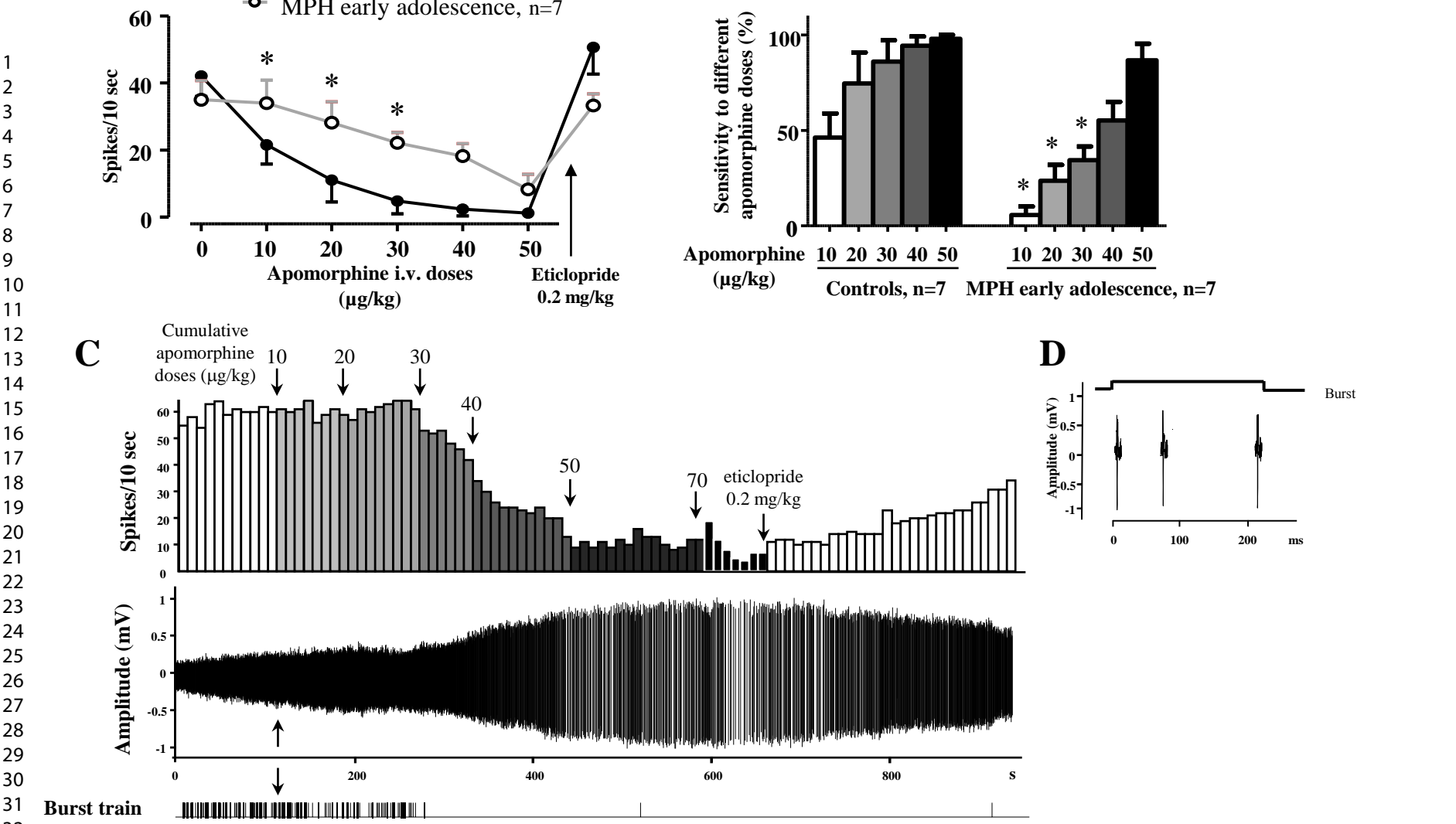


Figure 5: Early adolescent exposure to chronic methylphenidate induces partial desensitisation of dopamine neurons to apomorphine in adulthood.

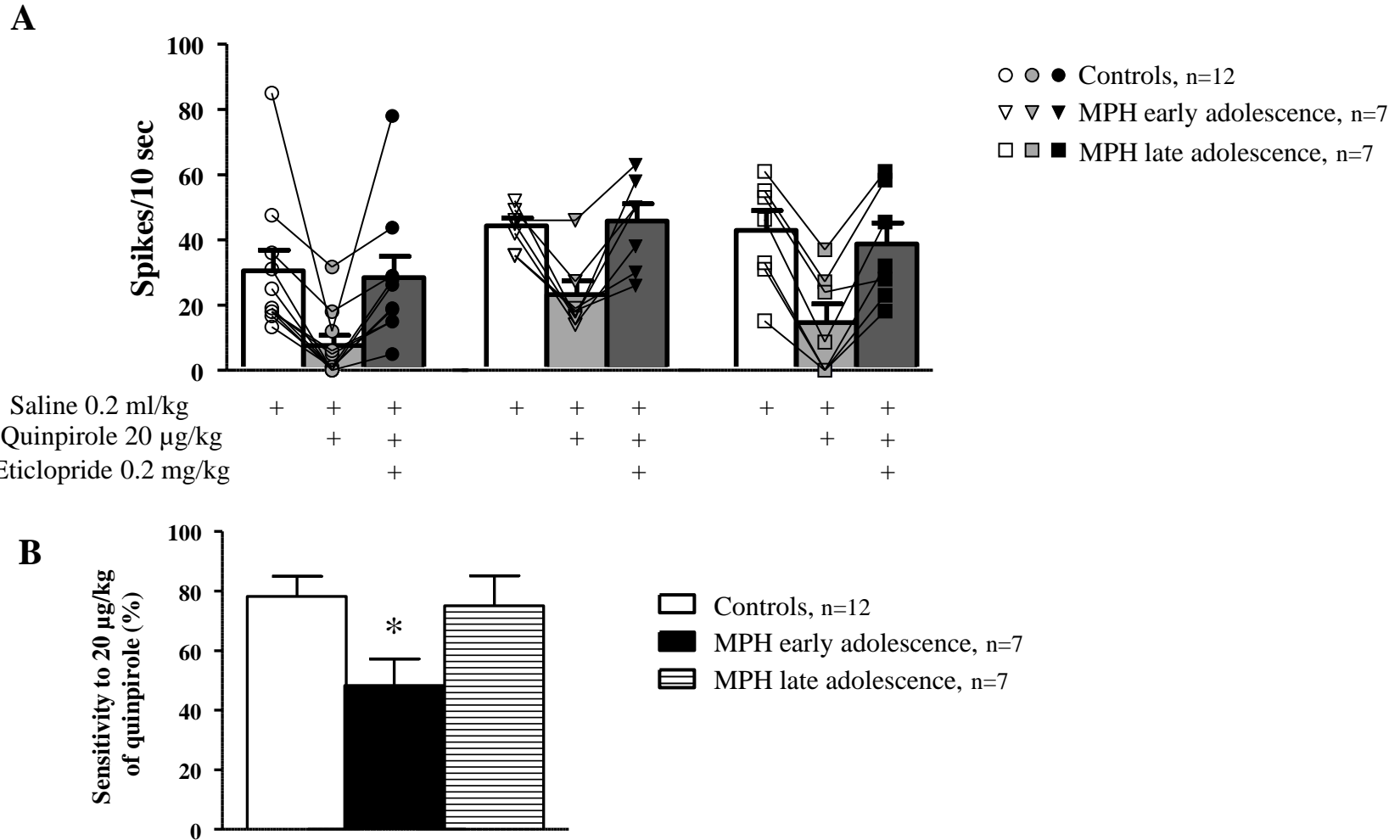


Figure 6: Early but not late adolescence exposure to chronic methylphenidate partially induces desensitisation of dopamine neurons to the D_2 agonist quinpirole in adulthood.

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Type of neurons	Treatment periods	Administration routes	Groups	Firing rates (spikes/ 10 sec)	Bursts (% of all spikes in burst)	n=
Serotonin neurons	Early-Adolescence	Sucrose solution	MPH	14.5 ± 1.9	14.7 ± 4.1**	50
			Vehicles	13.4 ± 1.3	3.6 ± 1.5	65
	Late-Adolescence	Sucrose solution	MPH	12.0 ± 1.1	8.6 ± 2.0	79
			Vehicles	15.2 ± 1.7	7.0 ± 3	33
Dopamine neurons	Early-Adolescence	Drinking water	MPH	38.5 ± 2.6	42.5 ± 3.7*	45
		Sucrose solution	MPH	39.3 ± 3.8	31.4 ± 5.4	24
			Vehicles	34.3 ± 3.8	26.5 ± 4.8	29
	Late-Adolescence	Drinking water	MPH	33.4 ± 5.2	26.7 ± 5.2 #	15
		Sucrose solution	MPH	26.5 ± 2.0	20.2 ± 2.9	74
			Vehicles	28.0 ± 1.8	20.2 ± 2.7	68
	Naive	n/a (water <i>ad libitum</i>)	Untreated	31.8 ± 3.9	28.7 ± 3.1	30

Table 1: Electrophysiological characteristics of dopamine neurons and serotonergic neurons according to periods of treatment and administration routes.